JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2004, p. 807–810 Vol. 42, No. 2
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.2.807–810.2004

NOTES

Emergence of Nonencapsulated and Encapsulated Non-b-Type Invasive *Haemophilus influenzae* Isolates in Portugal (1989-2001)

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Received 11 July 2003/Returned for modification 22 August 2003/Accepted 29 October 2003

Phenotypes and genetic relatedness of invasive *Haemophilus influenzae* strains were evaluated from 1989 through 2001. Among 119 isolates, multidrug resistance decreased (from 50 to 0%), the level of *H. influenzae* serotype b (Hib) strains declined (from 81 to 16%), the level of noncapsulated strains rose (from 19 to 80%), and the first invasive *H. influenzae* serotype f strain was described. This study documents changes in invasive *H. influenzae* infections in Portugal, i.e., the emergence of non-type-b strains that are genetically diverse and unrelated to Hib.

Haemophilus influenzae serotype b (Hib) was, until the introduction of a vaccine, responsible for most cases of invasive disease (10). Accurate antibiotic treatment is required as quickly as possible. The Hib vaccine was licensed in Portugal in 1994 and was recommended on a voluntary basis for children less than 5 years old. In 2000, the vaccine was included in the National Vaccination Plan. To our knowledge, there has been no study of invasive *H. influenzae* disease in this country. The aim of this study was to compare the phenotypes and genotypes of *H. influenzae* strains isolated from patients with invasive disease, according to the time of isolation, i.e., before the use of the Hib vaccine (1989 to 1993), during the period in which the vaccine was optional (1994 to 1999), and after the vaccine was included in the National Vaccination Plan (2000 to 2001).

(This work was presented in part at the 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, abstr. C2-1262, 2003.)

One hundred nineteen strains of *H. influenzae*, isolated between January 1989 and December 2001 from patients with invasive infections (52 from cerebral spinal fluid, 63 from blood, 4 from pleural fluid) at 16 hospitals in Portugal and sent to the Antibiotic Resistance Unit at the National Institute of Health in Lisbon, were included in this study. Phenotypic characterization of isolates was performed by standard procedures (3). PCR capsular genotyping was assessed in all non-b-type strains (53 strains) (8), and 56 strains (29 serotype b strains, 26 noncapsulated [NC] strains, and 1 serotype f strain) were randomly chosen for pulsed-field gel electrophoresis (PFGE) analysis (1). PFGE patterns were analyzed by using Bionumerics software (Applied Maths, Kortrijk, Belgium).

Overall, 72 of 119 (60.5%) strains were serotype b, 46 of 119 (38.6%) strains were NC, and 1 (0.8%) strain was type f. Fifty-eight invasive isolates were obtained from 1989 to 1993; 47 (81%) were serotype b and 11 (19%) were NC. Thirty-six strains were isolated from 1994 to 1999; 21 (58.3%) were serotype b and 15 (41.7%) were NC. Twenty-five strains were isolated from 2000 to 2001; 4 (16%) were serotype b, 20 (80%) were NC, and 1 (4%) was serotype f. Invasive strains were from biotypes I (66.4%), II (18.5%), IV (8.4%), III (5%), and V (1.7%). For biotype I strains, 57 of 79 (72.2%) were serotype b. The Hif strain was also biotype I. Only 73.1, 84, and 89.9% of the strains were susceptible to ampicillin, tetracycline, and chloramphenicol, respectively (Table 1). Three strains (2.5%) were classified as intermediate to rifampin (MIC, 2 μg/ml). Thirty-two strains (26.9%) were beta-lactamase producers. Some (34.4%) of the ampicillin-resistant strains were also resistant to chloramphenicol and tetracycline and were thus multidrug resistant (Table 2). Molecular analysis by PFGE revealed 20 unique genotypes (Fig. 1).

We show that the introduction of the vaccine in Portugal led to changes in *H. influenzae*, particularly the decline in strains of serotype b (from 81 to 16%), which was accompanied by a relative increase of NC strains (from 19 to 80%). We also report for the first time a Portuguese serotype f invasive strain that was isolated during the vaccination period. The efficacy of the Hib conjugate vaccine (10) has been extensively studied, and it significantly reduces the incidence of carriage in immunized children, which may have several consequences, e.g., the isolation of other serotypes in cases of invasive disease, especially serotypes a and f (18, 19); an increase in virulence of non-b serotypes (15, 16); and a concomitant increase in NC strains (9).

Multidrug resistance (ampicillin, chloramphenicol, and tetracycline) has decreased from 1989 to 2001 (from 50.0% in

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TABLE 1. MIC_{50}, MIC_{90}, MIC range, and MIC interpretation of 119 *H. influenzae* invasive isolates and the relationship with beta-lactamase production

Antimicrobial agent(s)	Isolates (*n*)[a]	MIC (μg/ml)[b]			% of isolates[c]		
		50%	90%	Range	Susceptible	Intermediate	Resistant
Ampicillin	All (119)	0.25	16	0.06–>64	73.1	2.5	24.4
	Bla^+(32)	8	64	2–>64		9.4	90.6
	Bla^- (87)	0.25	0.5	0.006–1	100		
Amoxicillin-clavulanic acid	All (119)	0.5	0.5	0.125–8	100		
	Bla^+ (32)	0.5	0.5	0.25–2	100		
	Bla^- (87)	0.25	0.5	0.125–1	100		
Cefotaxime	All (119)	0.0125	0.025	0.003–0.06	100		
	Bla^+ (32)	0.006	0.0125	0.003–0.06	100		
	Bla^- (87)	0.0125	0.025	0.003–0.025	100		
Ciprofloxacin	All (119)	0.0125	0.0125	0.003–0.12	100		
	Bla^+ (32)	0.0125	0.025	0.003–0.05	100		
	Bla^- (87)	0.0125	0.06	0.003–0.12	100		
Tetracycline	All (119)	1	8	0.125–64	84	3.4	12.6
	Bla^+ (32)	4	32	0.125–64	40.6	12.5	46.9
	Bla^- (87)	1	1	0.5–2	100		
Chloramphenicol	All (119)	0.5	1	0.25–16	89.9		10.1
	Bla^+ (32)	1	16	0.25–16	62.5		37.5
	Bla^- (87)	1	1	0.5–1	100		
Rifampin	All (119)	0.5	1	0.125–2	97.5	2.5	
	Bla^+ (32)	0.5	1	0.125–2	90.6	9.4	
	Bla^- (87)	0.5	1	0.5–1	100		

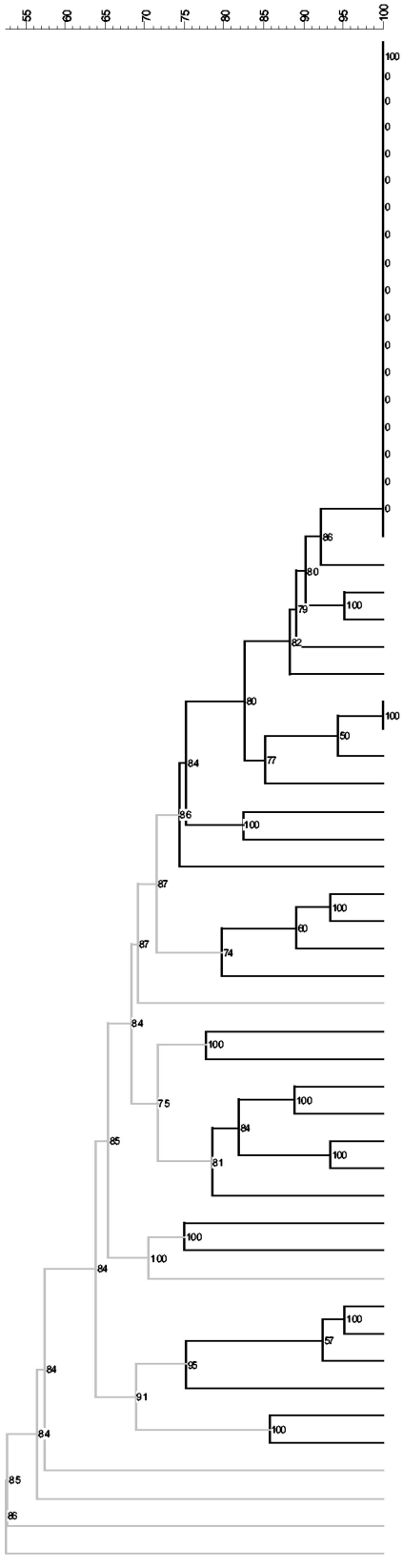
[a] Bla, beta-lactamase.
[b] 50% and 90%, MICs at which 50 and 90% of isolates are inhibited, respectively.
[c] Breakpoints according to NCCLS (14). Data are based on interpretation of MICs.

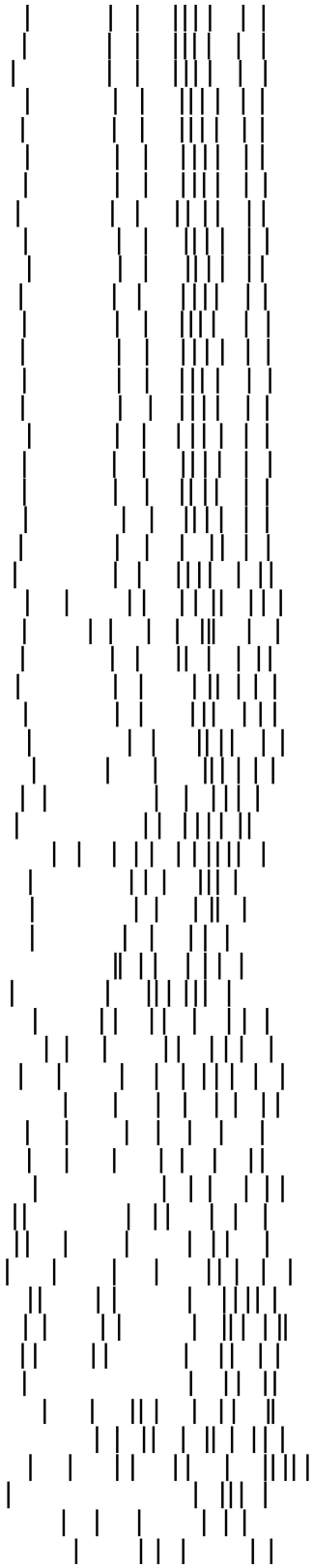
1989 to 1993, to 28.6% from 1994 to 1999, to 0% in 2000 and 2001). The decline of multidrug resistance appears to reflect the decline of Hib, as multidrug-resistant strains are mostly of serotype b (4). No beta-lactamase-negative ampicillin-resistant strains were detected during this study, a finding similar to that of a study of invasive infections in Brazil (5). However, the percentage of noninvasive NC strains in Portugal that are beta-lactamase negative and ampicillin resistant is increasing (P. Lavado and M. Caniça, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 59, 1999). None of our strains were resistant to amoxicillin-clavulanate, although the resistance mechanism for this drug combination seems to be increasing in some countries (7, 17). Cefotaxime or ceftriaxone seems to be an adequate treatment for meningitis and septicemia because *H. influenzae* strains that are resistant to these antibiotics have never to our knowledge been isolated before this study. The detection of strains with reduced susceptibility to rifampin indicates that it is important to continue monitoring susceptibility. Indeed, there have been resistant strains isolated in other countries (2, 17).

An analysis of genetic relatedness of invasive strains showed that 19 Hib strains, all but one of biotype I, seem to have the same clonal origin, being genetically identical and clustering into the major group at a genetic distance of 0%. It is possible that this is a single virulent clone, as both biotype I and Hib strains have long been associated with virulence (12). The remaining Hib strains are closely related. NC strains with PFGE pattern 0001 showed more than 80% similarity and seem to be genetically related to Hib strains with the same pattern. These NC clones were not strains that had lost the serotype b capsule because they were negative by PCR for the b type. However, there may have been evolutionary divergence from Hib strains involving mutations at the encapsulation locus. This finding may also suggest a partial loss of the *cap* locus; homologous recombination events in *H. influenzae* (11); or recombination events between *H. influenzae* and commensal flora, such as *Streptococcus mitis* (6), generating a new capsule type. The other NC strains did not cluster, except strains with PFGE patterns 0009 and 0014. These two clusters are genetically related to each other and may display clonal dissemination with the emergence of predominant clones in the future. The clone from serotype f was genetically unrelated to the Hib or NC invasive strains, as previously reported (13, 16, 19).

This study provides information about the diversity of inva-

FIG. 1. Dendrogram based on PFGE *Sma*I restriction pattern analysis using the unweighted pair group method of 56 *H. influenzae* isolates. The Dice band-based similarity coefficient, with a band position tolerance of 2.1% and an optimization of 1%, was used for clustering. A cutoff value of 80% similarity was determined by the cluster cutoff method according to Bionumerics software. Isolates with a dice band-based similarity coefficient value of >80% were considered to belong to the same cluster. Cophenetic correlations are shown in each branch of the dendrogram. From left to right are schematic restriction digest patterns, the strain code numbers, the pattern numbers, the biotypes, and the serotypes.





Strain code no.	Pattern no.	Biotype	Serotype
125	0001	I	b
262	0001	I	b
407	0001	I	b
829	0001	I	b
919	0001	I	b
922	0001	I	b
1209	0001	I	b
1578	0001	I	b
1909	0001	I	b
2018	0001	I	b
2048	0001	I	b
3449	0001	I	b
4784	0001	I	b
3448	0001	II	b
3514	0001	I	b
4111	0001	I	b
4359	0001	I	b
4419	0001	I	b
4527	0001	I	b
3665	0001	I	b
4786	0001	I	b
1449	0001	III	Nc
4816	0001	IV	Nc
4017	0001	II	b
1905	0001	I	b
4966	0001	I	b
2098	0001	I	b
4536	0001	I	Nc
594	0002	IV	Nc
4681	0002	I	b
4814	0003	I	Nc
2518	0004	I	b
4081	0004	IV	Nc
2057	0004	I	b
1219	0005	II	b
4729	0006	I	Nc
5745	0007	I	Nc
4104	0008	II	Nc
4099	0009	I	Nc
3533	0009	I	Nc
3023	0009	I	Nc
5387	0009	I	Nc
2104	0010	III	Nc
1402	0011	III	Nc
5308	0012	II	Nc
4565	0013	I	Nc
4896	0014	I	Nc
5446	0014	I	Nc
4230	0014	II	Nc
3227	0015	I	Nc
5709	0016	III	Nc
4866	0016	II	Nc
5768	0017	II	Nc
1358	0018	III	Nc
495	0019	I	Nc
5773	0020	I	f

TABLE 2. Resistance phenotype and serotype of the 32 ampicillin-resistant beta-lactamase producer strains

Yr of isolation (no. of strains)	Resistance phenotype	No. of strains (% multiresistant)	Serotype (no. of strains)
1989 (10)	Amp Chl Tet	4 (40)	b (4)
	Amp	1	b (1)
1990 (6)	Amp Chl Tet	2 (33.3)	b (2)
1991 (6)[a]			
1992 (22)	Amp	5	b (3), NC (2)
1993 (14)	Amp Chl Tet	1 (7.1)	b (1)
	Amp	1	NC (1)
1994 (5)	Amp Chl Tet	2 (40)	b (2)
	Amp Chl	1	b (1)
	Amp	2	b (1), NC (1)
1995 (3)	Amp Chl Tet	2 (66.7)	b (2)
	Amp	1	b (1)
1996 (1)	Amp Tet	1	b (1)
1997 (11)	Amp Tet	1	b (1)
	Amp	1	b (1)
1998 (6)[a]			
1999 (10)	Amp Tet	1	b (1)
	Amp	2	b (1), NC (1)
2000 (10)	Amp Tet	1	NC (1)
	Amp	1	NC (1)
2001 (15)	Amp	2	NC (2)

[a] All the strains from these years were susceptible to all the antibiotics tested.

sive *H. influenzae* clones in a Portuguese population and documents the emergence of NC genotypes. Few clones are responsible for invasive Hib disease (13). In contrast, the high levels of diversity and genetic heterogeneity of NC strains indicate different clonal origins, suggesting the introduction of new clones. Surveillance of *H. influenzae* isolated from cases of invasive disease will be required to monitor developments concerning this pathogen, as it is likely that non-serotype-b encapsulated *H. influenzae* or NC strains will emerge as vaccination becomes prevalent worldwide.

We thank Deolinda Louro and Susana Machado for their technical assistance in phenotypic determinations and Ricardo Dias for support in the construction of the dendrogram. The Antibiotic Resistance Unit of NIH thanks the following pharmaceutical companies represented in Portugal for having supplied antibiotics: Laboratórios Atral S.A., Carregado (ampicillin and tetracycline); GlaxoSmithKline, Algés (amoxicillin-clavulanate); Farma APS Produtos Farmacêuticos, Lisbon (cefotaxime); Edol Produtos Farmacêuticos, Linda-a-Velha (chloramphenicol); Aventis Pharma Lda, Mem-Martins (rifampin); and Bayer Portugal S.A., Carnaxide (ciprofloxacin).

This work was financially supported by grant PI 184/01 from Comissão de Fomento de Investigação em Cuidados de Saúde from the Ministério da Saúde and by the SDH.IC.I.01.11 grant from Fundação Calouste Gulbenkian, Lisbon, Portugal.

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